Title of Invention

Infectious cDNA clone of GB Virus B and Uses Thereof

Field of Invention

The present invention relates to nucleic acid sequence which comprises the genome of an infectious GB virus B (GBV-B) clone. The invention also relates to the use of the nucleic acid sequence of the infectious GB virus B clone to study indirectly the molecular properties of hepatitis C virus (HCV), and in the production of HCV/GBV-B chimeras. The invention further relates to the use of the infectious nucleic acid sequence of the GB virus B clone and the HCV/GBV-B chimeras in the development of vaccines and therapeutics for HCV.

Background of Invention

Transmission studies of potential human hepatitis agents were first reported in 1967 (Deinhardt 1967). Four tamarins inoculated with acute phase sera from a surgeon with acute hepatitis (patient GB) developed hepatitis, as did most tamarins inoculated in serial passage studies. Subsequent studies indicated that the etiological agent responsible for the development of hepatitis in these animals was not any of the known human hepatitis viruses (Purcell 1994). In 1995, two related RNA viruses named GB virus-B (GBV-B) and GB virus A (GBV-A) were identified in acute phase sera of a tamarin which developed hepatitis following inoculation with serum of the eleventh tamarin passage of the putative GB agent (Simons 1995a).

GBV-B infection of tamarins resulted in acute resolving hepatitis (Schlauder 1995, Buhk 1997). The

- 2 -

natural host of GBV-B is still unknown as the virus has
not been detected in uninoculated animals or in humans.

GBV-A, on the other hand, is an indigenous
tamarin virus rather than a component of the original GB
inoculum (Bukh 1997, Erker 1998). Experimental
infection of tamarins with GBV-A did not produce
hepatitis (Schlauder 1995). A human agent, GBV-C or
hepatitis G virus, most closely related to GBV-A, was
later identified (Simons 1995b, Linnen 1996). However,
it is still not clear whether this virus actually causes
hepatitis (Alter 1998, Bukh 1998a). Thus, of the known
GB viruses, GBV-B may be the only true hepatitis virus.

Based on analysis of their genomic sequences,
GBV-A, GBV-B and GBV-C were classified as members of the
Flaviviridae family of viruses, and among the known
viruses, GBV-B is the virus most closely related to
hepatitis C virus (HCV) (Muerhoff 1995, Robertson 1998).

The GBV-B virus contains a positive-sense,
single-stranded RNA genome of 9143 nucleotides (nts)
(Simons 1995a, Muerhoff 1995). The viral genome of GBV-
B consists of a 5' untranslated region (UTR), a single
long open reading frame (ORF) and a 3' UTR. Based on
known motifs, structural proteins were predicted to be
encoded in the 5' portion of the ORF and nonstructural
(NS) proteins in the 3' portion of the ORF (Muerhoff
1995). The hydropathy plots of the polyproteins of GBV-
B and HCV are very similar even though the overall
homology of the predicted polyproteins between GBV-B and
HCV is only about 25-30% (Muerhoff 1995). The putative
envelope proteins (E1 and E2) of GBV-B and HCV share
common structural features, and significant homology was
observed between the NS3 serine protease, the NS3 RNA

- 3 -

helicase, and the NS5 RNA-dependent RNA polymerase regions of GBV-B and HCV (Muerhoff 1995). Furthermore, the function and substrate specificity of the GBV-B and HCV NS3 serine proteases are also similar (Scarselli 1997). The genomic structure and organization of GBV-B and HCV share additional features of interest. First, colinear regions with significant sequence homology were identified in the 5' UTRs (Muerhoff 1995) and the predicted IRES structure of GBV-B is similar to that of HCV (Lemon 1997). Second, both viruses begin the 3' UTR with a short sequence followed by a poly (U) stretch followed by additional nucleotides (50 nucleotides for GBV-B and 98 nucleotides for HCV). However, the 3' terminal sequence of HCV forms a stable stem-loop structure (Kolykhalov 1996) whereas the published 3' terminal sequence of GBV-B does not.

To date, molecular studies of HCV are severely limited by the lack of an efficient cell culture system for the virus and by expense and limited availability of chimpanzees, the sole animal model for HCV. Accordingly, a less expensive and more readily available animal than chimpanzees is necessary as an animal model for the study of HCV.

Summary of Invention

The present invention relates to nucleic acid sequence which comprises the genome of an infectious GB virus B (GBV-B) clone. It is therefore an object of the invention to provide nucleic acid sequence which encodes an infectious GBV-B. Such nucleic acid sequence is referred to throughout the application as "infectious nucleic acid sequence".

As significant structural homology exists between the genomes of GBV-B and HCV, the invention also relates to the use of infection of tamarins with the infectious nucleic acid sequence of GBV-B or with mutants of the infectious sequence to study indirectly the molecular properties of hepatitis C virus (HCV) or as a preliminary screen to identify agents which have antiviral activity against HCV.

The invention further relates to "chimeric nucleic acid sequences" consisting of portions of the infectious nucleic acid sequence of GBV-B and portions of the nucleic acid sequences of other viruses closely related to GBV-B such as HCV, GBV-C or other members of the *Flaviviridae* family which do not replicate in tamarins. In a preferred embodiment, the chimeric nucleic acid sequences consist of portions of the infectious nucleic acid sequence of GBV-B and portions of the nucleic acid sequence of HCV. The nucleic acid sequences taken from GBV-B and HCV can be open-reading frame sequences, and/or sequences from the 5'UTR and/or 3'UTR.

In one embodiment, GBV-B/HCV chimeras may be made in which 5' or 3' UTR sequences of the GBV-B infectious clone are replaced with the corresponding sequence from an HCV clone.

In another embodiment, GBV-B/HCV chimeras may be constructed in which the structural or non-structural regions of GBV-B are replaced by corresponding regions of HCV. Thus, such a chimera would contain, for example, the HCV structual region in a GBV-B "genomic backbone". Of course, it is understood by one of skill in the art that the construction of the above-described

- 5 -

chimeric nucleic acid sequences may be reversed such
that, for example, the GBV structural region may replace
the structual region of an HCV genome to produce a
chimera in which the GBV structural region is contained
in an HCV backbone.

The invention further relates to the use of
the chimeric nucleic acid sequences of the invention to
study the functions of HCV genes, and for the
development of vaccine and antiviral agents against HCV.

The invention also relates to the use of the
infectious GBV-B nucleic acid sequence, the mutated GBV-
B nucleic acid sequences or the chimeric sequences of
the invention to identify cell lines capable of
supporting the replication of GBV-B or the chimeras of
the invention.

The present invention also relates to the
polypeptides encoded by the nucleic acid sequences of
the invention or fragments thereof.

The present invention further relates to the
in vitro and in vivo production of GBV-B, mutant GBV-B
viruses or chimeric GBV-B/HCV viruses from the nucleic
acid sequences of the invention.

The invention also provides that the nucleic
acid sequences and viruses of the invention be supplied
in the form of a kit, alone or in the form of a
pharmaceutical composition.

Brief Description Of Figures

Figure 1 shows a flow diagram of GB virus
transmission studies in two species of tamarins,
Saguinus mystax (SM) and Saguinus oedipus (SO). The
animals infected with GBV-B (Simons 1995a) are boxed.

- 6 -

Two serum pools (GB 8/93 and GB 2/94) were made from
acutely infected animals. Both pools contained GBV-B,
as well as GBV-A (Simons 1995) at a titer of 10^8 genome
equivalent (GE)/ml. A 10% liver homogenate (CT 11/91)
was made from a sacrificed tamarin. A number of *S.
mystax* tamarins (SM 737, 749, 750, 760, 782, 795 and
799) and *S. oedipus* tamarins (SO 100) were naturally
infected with GBV-A_{SM} and GBV-A_{SO}, respectively, prior to
inoculation (Bukh 1997). Only two tamarins (SM 720 and
748), both GBV-A_{SM} negative, became infected with GBV-A
(Simons 1995) following inoculation. Tamarins SM42 and
SM670 were not tested for GBV-A or GBV-A_{SM}.

Figure 2 shows the course of GBV-B infection in
tamarins (*S. mystax*) inoculated with a dilution series of
the GB 2/94 pool. All animals were inoculated
intravenously at week 0 with 1 ml of the indicated
dilution. Results of qualitative RT-nested PCR for GBV-B
in serum are shown at the top (filled circles, positive;
empty circles, negative). Serum levels of isocitrate
dehydrogenase (ICD in units/ml); shaded area) and the
estimated $\log_{10}$ GBV-B GE titer (vertical columns) were
plotted against time.

Figure 3 shows alignment of the 3' UTR
sequences of GBV-B. The sequence of the infectious clone
of GBV-B (pGBB) is shown at the top (nts. 9038-9399).
The other sequences shown are: pGBB5-1, a non-infectious
clone of GBV-B; GBV-B, a prototype of GBV-B (Simons
1995); eleven "gb" clones obtained from CT 11/91 liver
homogenate by 5' RACE on the minus-strand GBV-B RNA; four
"29" clones obtained from GB 2/94 pool by RT-PCR across
5'-to-3'-end-ligated viral GBV-B RNA; and seven "GBB3"
clones obtained from GB 2/94 pool by standard RT-PCR.

- 7 -

With pGBB as the reference, nucleotide substitutions or insertions are shown as uppercase letters, identical nucleotides are shown as dots and nucleotide deletions are shown as dashes.

Figure 4 shows the predicted secondary structure of the 3' UTRs of GBV-B and HCV as determined by the program "mfold" (Genetics Computer Group).

Figure 5 shows the course of GBV-B infection in *S. mystax* tamarins transfected with RNA transcripts of pGBB. Both animals were negative for GBV-A$_{SM}$. At week 0 transcription mixtures were injected into tamarins by percutaneous intrahepatic injection guided by ultrasound. Results of qualitative RT-nested PCR for GBV-B in serum is shown at the top (filled circles, positive; empty circles, negative). Serum levels of isocitrate dehydrogenase (ICD in units/ml; shaded area) and the estimated $\log_{10}$ GBV-B GE titer (vertical columns) were plotted against time.

Figures 6A-6F show the nucleotide sequence of the infectious hepatitis C virus clone of genotype 1a strain H77C and Figures 6G-6H show the amino acid sequence encoded by the clone.

Figures 7A-7F show the nucleotide sequence of the infectious hepatitis C virus clone of genotype 1b strain HC-J4 and Figures 7G-H show the amino acid sequence encoded by the clone.

Description of The Invention

The present invention relates to nucleic acid sequence which comprises the genome of an infectious GB virus B (GBV-B) clone. The nucleic acid sequence which comprises the genome of an infectious GBV-B virus is

- 8 -

shown in SEQ ID NO:1 and is contained in the plasmid
construct pGBB deposited with the American Type Culture
Collection (ATCC) on May 28, 1999 and having ATCC
accession number PTA-152. The present invention relates
to the identification of a 260 nucleotide sequence at
the 3' end of the infectious GBV-B clone which is shown
in Example 3 to be necessary for the development of the
infectious clone.

Since GBV-B is the virus most closely related
to HCV, the present invention also relates to
experimental infection of tamarins with the infectious
GBV-B clone of the invention or with mutants of the
infectious GBV clone to study indirectly the molecular
properties of HCV or as a preliminary screen to identify
agents which have antiviral activity against HCV. For
example, since the predicted internal ribosome entry
site (IRES) structure in the 5'UTR of GBV-B is similar
to that of HCV (Lemon 1997), the NS3 serine proteases of
GBV-B and HCV have been shown to share substrate
specificity in vitro (Scarselli 1997), and the 3'UTRs of
HCV (Yanagi 1999) and GBV-B (see Examples) have been
shown to be critical for viral infectivity, mutagenesis
of these regions in the GBV-B infectious clone may be
undertaken to examine IRES function, NS3 serine protease
activity or the role of the 3'UTR in viral infectivity
in vivo. Where such "mutations" are introduced into the
GBV-B clone of the invention to create a "mutated" GBV-B
sequence, the mutations include, but are not limited to,
point mutations, deletions and insertions. Of course,
one of ordinary skill in the art would recognize that
the size of the insertions would be limited by the
ability of the resultant nucleic acid sequence to be

- 9 -

properly packaged within the virion. Such mutations could be produced by techniques known to those of skill in the art such as site-directed mutagenesis, fusion PCR, and restriction digestion followed by religation.

Alternatively, given the significant structural homology that exists between the genomes of GBV and HCV, the infectious GBV-B clone may be used to screen for inhibitors of IRES function or viral enzyme activity (for example, NS3 helicase, NS3 protease, NS2-NS3 protease or NS5B RNA polymerase activity). Such inhibitors may be useful as antiviral agents to HCV since viral enzyme activity and IRES function are known to be critical for HCV replication.

The effect of such inhibitors on the IRES function or viral activity of the GBV-B encoded by the infectious sequence of the invention may be measured by assays known to those of skill in the art to measure directly or indirectly viral replication or viral pathogenicity. Such assays include, but are not limited to, the measurement of virus titer in serum or liver of an infected tamarin by PCR or the measurement of GBV-B viral protein expression in liver cells of an infected tamarin by immunoflourescence or Western blot. Of course, it is understood that a comparison of results obtained for control tamarins (treated only with infectious nucleic acid sequence) with those obtained for treated tamarins (nucleic acid sequence and antiviral agent) would indicate, the degree, if any, of antiviral activity of the candidate antiviral agent. Of course, one of ordinary skill in the art would readily understand that the tamarins can be treated with the candidate antiviral agent either before or after

- 10 -

exposure to the infectious nucleic acid sequence of the
present invention.

        In yet another embodiment, the invention
relates to "chimeric nucleic acid sequences" which
consist of portions of the infectious nucleic acid
sequence of GBV-B and portions of nucleic acid sequences
of viruses which are related to GBV-B such as HCV, GBV-C
and other members of the Flaviviridae family which do
not infect tamarins. In a preferred embodiment,
chimeric nucleic acid sequences consist of portions of
the infectious nucleic acid sequence of GBV-B and
portions of nucleic acid sequences of hepatitis C
viruses (HCV) of various genotypes or subtypes;
preferably portions of nucleic acid sequence of
infectious HCV clones of genotypes 1a (ATCC accession
number PTA-157; Figures 6A-6F), 1b (ATCC accession
number 209596; Figures 7A-7F) or 2a (ATCC accession
number PTA-153; SEQ ID NO: 4). The nucleic acid
sequences taken from GBV-B and HCV can be open-reading
frame sequences, and/or sequences from the 5'UTR and/or
3'UTR. The gene borders of the HCV genome, including
nucleotide and amino acid locations, have been
determined, for example, as depicted in Houghton, M.
(1996), and the putative gene borders of the GBV-B are
shown in Table 1.

        Of course, it is understood that the
production of GBV-B/HCV chimeras could include insertion
of specific genes or regions of the infectious GBV-B
clone into an HCV "genomic backbone" (where the HCV
genomic backbone is preferably an infectious nucleic
acid sequence of HCV genotypes 1a, 1b or 2a described
above) or alternatively, could include insertion of

specific genes (or portions thereof) or regions of an HCV genome into the GBV-B infectious clone of the invention. Of course, where HCV genes or regions are to be inserted into the GBV-B infectious clone, it is to be understood that the inserted HCV sequences may be unmodified or may be mutated in order to examine the effect of the mutation(s) on the function of the inserted HCV gene or region in the chimeric GBV-B-HCV virus.

Such chimeras can readily be produced by methods known to those of ordinary skill in the art.

In one embodiment, GBV-B/HCV chimeras may be made in which 5' or 3' UTR sequences of the GBV-B infectious clone are replaced with the corresponding sequence from an HCV clone. For example, chimeras may be constructed in which the IRES sequence of the infectious GBV-B clone is replaced by the IRES sequence of HCV. Such chimeras can be used in identifying inhibitors of IRES activity which would be useful as antiviral agents, or could be used to examine HCV IRES function in vivo. Alternatively, mutations could be introduced into the HCV IRES contained in the GBV-B clone in order to examine the effect of the mutation(s) on IRES function in vivo.

Alternatively, GBV-B/HCV chimeras may be made in which the 3'UTR sequence of GBV-B is replaced by the 3'UTR sequence of HCV. As the 3' terminal stem-loop structure is believed to be important for initiation of RNA replication and has been shown to be critical for infectivity of HCV in vivo, such chimeras may be used for more detailed analysis of the function of the 3' UTR

- 12 -

sequence of HCV in vivo and for the testing of candidate antiviral agents.

In another embodiment, GBV-B/HCV chimeras may be constructed in which the structural or non-structural regions of GBV-B are replaced by corresponding regions of HCV. Such chimeras would be useful in identifying whether the inability of HCV to infect tamarins is due to the inability of HCV's structural region to bind the receptor necessary for infection of tamarins or to the absence of sequences in HCV's nonstructural regions which are necessary for replication in tamarins. For example, the ability to infect tamarins with GBV-B/HCV chimeras in which the non-structural region of GBV-B is replaced by the non-structural region of HCV would indicate that the structural genes of GBV-B are necessary for viral infection in tamarins, and that the inability of HCV to infect tamarins is likely due to its lack of receptors for HCV.

Alternatively, the ability to infect tamarins with GBV-B/HCV chimeras in which the structural region of GBV-B is replaced by the structural region of HCV would indicate that the non-structural genes of GBV-B are critical for viral infection in tamarins, and that the inability of HCV to infect tamarins is likely due to HCV's lack of nonstructural sequences which are necessary for replication in tamarins.

Of course, GBV-B-HCV chimeras may be constructed in which only a portion of the non-structural or structural regions of GBV-B are replaced by the corresponding portions of HCV sequences. For example, a chimera in which only one or two of the three structural genes (C, E1 and E2) of GBV-B are replaced by

- 13 -

the corresponding HCV structural genes may be made. In
one embodiment, nucleic acid sequences comprising the E1
and E2 genes of GBV-B may be replaced by the sequences
comprising the HCV E1 and E2 genes. In another
embodiment, nucleic acid sequence comprising either the
E1 or E2 gene of GBV-B is replaced by sequence encoding
either the HCV E1 or E2 gene.

Alternatively, only a fragment of a GBV-B
structural gene in the infectious GBV clone may be
replaced with the corresponding HCV gene fragments. For
example, the amino terminal of the GBV-B E1 gene may be
replaced by the corresponding portion of an HCV E1 gene
or an amino terminal portion of the GBV-B E2 gene may be
replaced by an amino terminal portion of HCV E2 gene tht
containing the HVR1 region. As the structural genes of
HCV are believed to be important for neutralization,
chimeras containing an HCV structural gene(s) or
fragment(s) thereof can be used to develop vaccines
against HCV.

In yet another embodiment, chimeras in which
individual non-structural genes of GBV-B, such as NS3
RNA helicase, NS3 protease, or the NS5B RNA-dependent
RNA polymerase are replaced by the corresponding non-
structural genes of HCV may be constructed. Such
chimeras would, for example, be useful in identifying
inhibitors of viral enzyme activity which would be
useful as antiviral agents. Of course, it is understood
that in order to construct chimeras in which the
polyprotein cleavage sites of the GBV-B remain intact,
it may be desirable to replace only a fragment of a
nonstructural gene of GBV-B with the corresponding HCV
gene fragment.

- 14 -

The present invention also relates to polypeptides encoded by the nucleic acid sequences of the invention or fragments thereof. In one embodiment, said polypeptide or polypeptides may be fully or partially purified from viruses produced by cells transfected with the nucleic acid sequences of the invention. In another embodiment, the polypeptide or polypeptides may be produced recombinantly from a fragment of the nucleic acid sequences of the invention. In yet another embodiment, the polypeptides may be chemically synthesized.

The present invention further relates to the in vitro and in vivo production of GBV-B, mutated GBV-B or chimeric GBV-B/HCV viruses from the nucleic acid sequences of the invention.

In one embodiment, the sequences of the invention can be inserted into an expression vector that functions in eukaryotic cells. Eukaryotic expression vectors suitable for producing high efficiency gene transfer in vivo are well known to those of ordinary skill in the art and include, but are not limited to, plasmids, vaccinia viruses, retroviruses, adenoviruses and adeno-associated viruses.

In another embodiment, the sequences contained in the recombinant expression vector can be transcribed in vitro by methods known to those of ordinary skill in the art in order to produce RNA transcripts which encode the GBV-B of the invention. The GBV-B of the invention may then be produced by transfecting cells by methods known to those of ordinary skill in the art with either the in vitro transcription mixture containing the RNA

- 15 -

transcripts or with the recombinant expression vectors
containing the nucleic acid sequences described herein.

In assaying the ability of the mutated GBV-B
sequences or of the chimeric sequences of the invention
to infect tamarins, the virulence phenotype of the virus
produced by transfection of tamarins with the sequences
of the invention can be monitored by methods known in
the art such as measurement of liver enzyme levels
(alanine aminotransferase (ALT) or isocitrate
dehydrogenase (ICD)) or by histopathology of liver
biopsies.

The present invention also relates to the use
of the infectious GBV-B sequence, the mutated GBV-B
nucleic acid sequences or the chimeric sequences of the
invention to identify cell lines capable of supporting
the replication of GBV-B or the chimeras of the
invention.

Transfection of tissue culture cells with the
nucleic acid sequences of the invention may be done by
methods of transfection known in the art such as
electroporation, precipitation with DEAE-Dextran or
calcium phosphate, or incorporation into liposomes.

In one such embodiment, the method comprises
the growing of animal cells in vitro and transfecting
the cells with the nucleic acid of the invention, then
determining if the cells show indicia of GBV-B or HCV
infection. Such indicia include the detection of viral
antigens in the cell, for example, by immunofluorescence
procedures well known in the art; the detection of viral
polypeptides by Western blotting using antibodies
specific therefor; and the detection of newly
transcribed viral RNA within the cells via methods such

- 16 -

as RT-PCR. The presence of live, infectious virus particles following such tests may also be shown by injection of cell culture medium or cell lysates into healthy, susceptible animals, with subsequent exhibition of the signs and symptoms of GBV-B infection.

Suitable cells or cell lines for culturing GBV-B or the chimeric GBV-B-HCV include, but are not limited to, lymphocyte and hepatocyte cell lines known in the art.

Alternatively, primary hepatocytes can be cultured, and then infected; or, the hepatocyte cultures could be derived from the livers of infected tamarins. In addition, various immortalization methods known to those of ordinary skill in the art can be used to obtain cell-lines derived from hepatocyte cultures. For example, primary hepatocyte cultures may be fused to a variety of cells to maintain stability.

The invention also provides that the nucleic acid sequences and viruses of the invention be supplied in the form of a kit, alone or in the form of a pharmaceutical composition.

All scientific publication and/or patents cited herein are specifically incorporated by reference. The following examples illustrate various aspects of the invention but are in no way intended to limit the scope thereof.

EXAMPLES

Materials and Methods

Source of GB virus B

Two tamarin pools VR-806, (American Type Culture Collection) and H205, were used for experimental

- 17 -

transmission of the GB virus agents to tamarins species
Saguinus mystax and *Saguinus oedipus*.

Amplification, cloning and sequence analysis of GBV-B

Viral RNA was extracted from aliquots of the
GB 2/94 serum pool or CT 11/91 liver homogenate with the
TRIzol system (GIBCO/BRL). Primers used in cDNA
synthesis and PCR amplification were based on the
genomic sequence of GBV-B published by Simons et al
(Simons 1995) shown in SEQ ID NO:3. Long RT-PCR was
performed using Superscript II reverse transcriptase
(GIBCO/BRL) and the Advantage cDNA polymerase mix
(Clontech) as described previously (Tellier 1996). Four
subgenomic regions of GBV-B covering the entire
published sequence (Simons 1995) were amplified from
serum and the PCR products were purified and cloned into
pGEM-9Zf(-) (Promega) or pCR2.1 vector (Invitrogen)
using standard procedures.

The 5' terminus of GBV-B was amplified from
serum by using the rapid amplification of cDNA ends
(RACE) with dC or dA tailing (GIBCO/BRL) and GBV-B
specific antisense primers. Two different approaches
were used to determine the 3' terminal sequence of GBV-
B. In one approach, GBV-B RNA extracted from serum was
circularized with T4 RNA ligase (Promega) and the 5'-to-
3'-end-ligated viral RNA was amplified in RT-PCR using
specific GBV-B primers. In the second approach, the 5'
end of the negative strand GBV-B RNA extracted from the
liver homogenate was amplified using the 5' RACE with dC
tailing and GBV-B specific sense primers. The PCR
products were cloned directly into pCR2.1-TOPO by using
the TOPO TA Cloning Kit (Invitrogen).

- 18 -

The consensus sequence of GBV-B was determined
by direct sequencing of PCR products (nucleotides 1-9078
and nucleotides 9130-9359) and by sequence analysis of
the clones (nucleotides 1-7135 and nucleotides 7151-
9399). Nucleotide positions correspond to those of the
infectious clone (pGBB). Analyses of genomic sequences
were performed with GeneWorks (Oxford Molecular Group)
(Bukh 1995). To determine whether the GenBank data base
contained sequences with homology to the GBV-B 3' UTR
sequence identified in the present invention, a "Blast"
search was performed. The predicted secondary structure
of the GBV-B and HCV 3' UTR sequences were determined by
the program "mfold" (Genetics Computer Group).

Construction of consensus cDNA clones of GBV-B

First, clone pGBB5-1, a consensus clone of
GBV-B 2/94 containing the 3' terminus of GBV-B as
published by Simons *et al* was constructed (Simons
1995a). The core sequence of the T7 promoter, a 5'
guanosine residue and the sequence of GBV-B (9139
nucleotides) were cloned into pGEM-9Zf(-) vector using
NotI/SacI sites. A *BamHI* site was included at the GBV-B
3' terminus. Digested fragments containing the
consensus sequence were purified from subclones and
ligated using convenient sites. Next, a second
consensus clone of GBV-B, clone pGBB, was constructed by
inserting the additional 3' terminal sequence, amplified
by PCR from one of the clones obtained by the RACE
procedure described above, into pGBB5-1 using *XmaI* (at
position 9114) and *BamHI* sites. A *XhoI* site was
inserted following the GBV-B 3' terminus. DH5-alpha
competent cells (GIBCO BRL) were transformed and
selected on LB agar plates containing 100 µg/ml

- 19 -

ampicillin (SIGMA) and amplified in LB liquid cultures
at 30°C for 18-20 hrs (Yanagi 1997). Each cDNA clone
was re-transformed to select a single clone, and
large-scale preparation of plasmid DNA was performed
with a QIAGEN plasmid Maxi kit as described previously
(Yanagi 1997). Each clone was genetically stable since
the digestion pattern was as expected following
retransformation and the complete sequence was the
expected one.

Intrahepatic transfection of tamarins with transcribed
GBV-B RNA

In 100 µl reactions, RNA was transcribed *in
vitro* with T7 RNA polymerase (Promega) from 10 µg of
linearized template plasmid. The plasmid pGBB5-1 was
linearized with *BamHI* (Promega) and the plasmid pGBB was
linearized with *XhoI* (Promega). The integrity of the
RNA was checked by electrophoresis through agarose gel
stained with ethidium bromide. Each transcription
mixture was diluted with 400 µl of ice-cold
phosphate-buffered saline without calcium or magnesium
(SIGMA) and then immediately frozen on dry ice and
stored at -80°C. Within 24 hours of synthesis, two
transcription mixtures were injected into each tamarin
by percutaneous intrahepatic injection guided by
ultrasound (Emerson, 1992; Yanagi 1998, 1999). If the
tamarin did not become infected, the same transfection
was repeated once. All transfected animals were
negative for GBV-A$_{SM}$ as determined by the protocol
described previously (Bukh 1997a).

Monitoring of experimental course in tamarins

- 20 -

Serum samples were collected weekly from the
tamarins and monitored for liver enzyme levels [alanine
aminotransferase (ALT), gamma-glutamyltranspeptidase
(GGT), and isocitrate dehydrogenase (ICD)] by standard
methods and for GBV-B RNA by a specific reverse
transcriptase-polymerase chain reaction (RT-PCR) assay.
Total RNA was extracted from 100 µl of serum using the
TRIzol reagent. The RNA pellet was resuspended in 10 mM
dithiothreitol (DTT) containing 5% (vol/vol) of RNasin
(20-40 u/µl) (Promega). The RT-nested PCR was performed
with primers from the 5' UTR of GBV-B (external primer
pair: 5'-CCT AGC AGG GCG TGG GGG ATT TCC-3' and 5'-AGG
TCT GCG TCC TTG GTA GTG ACC-3'; internal primer pair:
5'-GGA TTT CCC CTG CCC GTC TG-3' and 5'-CCC CGG TCT TCC
CTA CAG TG-3'). The reverse transcription was performed
with avian myeloblastosis virus reverse transcriptase
(Promega) and the external anti-sense primer and nested
PCR was performed with AmpliTaq DNA polymerase or
AmpliTaq Gold DNA polymerase (Perkin Elmer) as described
previously (Bukh 1998a). Specificity was confirmed by
sequence analysis of selected DNA products. Each set of
experiments included a positive control sample (a 10^{-6}
dilution of GB 8/93, estimated titer 100 genome
equivalent (GE)) and appropriate negative control
samples. The genome equivalent (GE) titer of GBV-B in
positive samples was determined by RT-nested PCR on
10-fold serial dilutions of the extracted RNA (Bukh
1998a). One GE was defined as the number of GBV-B
genomes present in the highest dilution positive in RT-
nested PCR. The sensitivity of this RT-nested PCR assay
for GBV-B was equivalent to that of our RT-nested PCR
assay for HCV (Bukh 1998b), for example, conserved NS3

- 21 -

primers which had the same sensitivity for GBV-B as the 5' UTR primers could detect HCV at optimal sensitivity in samples with known HCV genome titer. Testing for GBV-A and GBV-A variants was performed by RT-nested PCR assays as described previously (Bukh 1997a).

The consensus sequence of the complete ORF was determined by direct sequencing of overlapping PCR products obtained by long RT-nested PCR on serum from one of the tamarins infected with RNA transcripts as previously described (Yanagi 1997).

Example 1

Transmission of GB Agent in Tamarins

To generate virus pools of the GB agent, tamarins were inoculated intravenously with pooled sera of the eleventh tamarin passage of this agent (Fig. 1). Acute phase sera from a *S. mystax* tamarin which developed hepatitis were pooled (GB 8/93) and inoculated into additional *S. mystax* tamarins to generate a second pool of acute phase serum (GB 2/94). Both serum pools contained approximately 10^8 GE/ml of GBV-B and GBV-A. A 10% liver homogenate (CT 11/91) was prepared from a *S. oedipus* tamarin which developed hepatitis following inoculation with the twelfth passage of the GB agent. The titer of GBV-B in the liver homogenate was approximately 10^7 GE/ml. The GB 2/94 serum and CT 11/91 liver samples were used as GBV-B cloning sources in the present study.

Inoculation of eight *S. mystax* tamarins with ten-fold serial dilutions of the GB 2/94 pool demonstrated that its infectivity titer of GBV-B was 10^8 tamarin 50% infectious doses (TID_{50}) (Fig. 2). The five

- 22 -

GBV-B infected tamarins all developed acute resolving
hepatitis characterized by early appearance of viremia
(weeks 1 or 2 p.i.), peak viral titers of 10^7-10^8 GE/ml
and clearance of viremia after 9-16 weeks (Fig. 2). Two
of these tamarins (*S. mystax* 769 and 777) were infected
only with GBV-B and were negative for GBV-A and GBV-A$_{SM}$,
whereas the other three tamarins were infected with both
GBV-B and GBV-A$_{SM}$. A *S. mystax* tamarin inoculated with
the liver homogenate also developed acute resolving
hepatitis with peak GBV-B titers of 10^7 GE/ml and
clearance of viremia after 11 weeks. Likewise, four *S.
mystax* tamarins inoculated with dilutions of the GB 8/93
pool developed acute resolving hepatitis with clearance
of the GBV-B virus after 11-26 weeks. Thus, GBV-B
infection in *S. mystax* tamarins is characterized by
acute hepatitis, early appearance of viremia, high peak
viral titers and viral clearance.

Example 2

Novel 3' Terminal Sequence of GBV-B

The consensus sequence of the complete 5' UTR
of GBV-B (nucleotides 1-445) was deduced from 13 clones
containing nucleotides 1-283 and 3 clones containing
nucleotides 31-445. In addition, the entire 5' UTR
sequence was determined by direct sequencing of the
amplicons. The sequences of the various clones were
highly conserved and the consensus 5' UTR sequence of
GBV-B from this pool was identical to that of the
previously published sequence for GBV-B (Simons 1995a).
It is noteworthy that 13 of 15 clones analyzed from the
rapid amplification of cDNA ends (RACE) procedure
contained the published GBV-B 5' terminus (A residue)

- 23 -

and that the same 5' terminus was obtained whether the 5' RACE was performed with dC or dA tailing.

The consensus sequence of the ORF (nucleotides 446-9037) was determined by direct sequencing of PCR products obtained using long RT-PCR (Yanagi 1997). In addition, 3 clones containing nts. 446-7135 (one of these clones had a deletion of nts. 3036-3636), 2 clones containing nts. 2019-3373, 5 clones containing nts. 7151-8261 and 7 clones containing nts. 7521-9037 were analyzed. The sequences of GBV-B clones in this pool were very homogeneous. Evidence of micro-heterogeneity was found at only 70 (0.8%) nucleotide and 36 (1.3%) amino acid positions, scattered throughout the ORF. The proportion of amino acid positions with heterogeneity ranged from 0.5-3.2% in different putative gene regions (lowest in NS3 and NS5B; highest in E2 and NS2). The GBV-B ORF sequence differed from the published sequence of GBV-B (Simons 1995) at 34 (0.4%) nucleotide and 12 (0.4%) deduced amino acid positions, respectively (Table 1).

- 24 -

Table 1

Nucleotide and amino acid differences among GBV-B (Simons 1995a), the consensus sequence of GBV-B recovered from a virus pool used as the cloning source (GBV-B, 2/94) and the infectious clone of GBV-B (pGBB).

Genomic Region*	Position nt [aa]	Nucleotide			Amino Acid		
		GBV-B	GBV-B 2/94	pGBB	GBV-B	GBV-B 2/94	PGBB
5' UTR (1-445)							
C (446-913)							
E1 (914-1489)	1030	C	T	T			
E2 (1490-2641)	1498	T	C (t)	C			
	1628 [395]	G	A (g)	A	V	I (V)	I
	2552 [703]	G	A (g)	A	D	N (D)	N
	2562,2563 [706]	C,A	A,C	A,C	P	H	H
	2566	T	T	T			
	2625 [727]	C	T	T	A	V	V
NS2 (2642-3385)	2647	C	T (c)	T			
	2816 [791]	C	T	T	L	F	F
	2855 [804]	A	G	G	T	A	A
	3235	A	G	G			
NS3 (3386-5125)	3475**	C	C (t)	T			
	3760	C	T (c)	T			
	4114	C	T	T			
	4117	C	A	A			
	4177	T	C	C			
	4615	C	T	T			
NS4A (5126-5290)							
NS4B (5291-6034)	5329	C	T	T			
	5332	T	C	C			
	5350	A	C	C			
	5455	C	T (c)	T			
NS5A (6035-7267)	6413 [1990]	T	A (t)	A	L	M (L)	M
	6577	G	T	T			
	6690 [2082]	T	C (t)	C	I	T(I)	T
	6965 [2174]	T	C (t)	C	S	P (S)	P
	7015	A	G (a)	G			
	7128 [2228]	G	A	A	G	E	E
	7138**	A	A	G			
	7142 [2233]	A	G	G	T	A	A
NS5B (7268-9037)	7282	T	C (t)	C			
	7849	C	A	A			
	7852	C	T	T			
	8942 [2981]	G	A (g)	A	V	I (V)	I
	8971	T	C	C			
	9026	C	T (c)	T			
3' UTR (9038-9399)	9067	T	C	C			
	Poly(U)	27 nts	11-23 nts	23 nts			
	9134	Deletion	C	C			
	9141-9399	ND	259 nts	259 nts			

*Nucleotide positions corresponding to pGBB. Putative gene borders defined as suggested by homology with HCV (Muerhoff 1995). No homology was observed at the NS2-NS3 junction.
**Positions that differ between the cloning source (GBV-B 2/94) and the infectious clone of GBV-B (pGBB). The change introduced into pGBB at position 7138 introduced an artificial SalI site. nd: Not determined. Nucleotides and amino acids shown in parenthesis were found as a minor species in the cloning source (GBV-B, 2/94

- 25 -

The sequence for the 3' UTR is shown in Figure
3.   Additional 3' UTR sequence was initially identified
by performing RT-PCR across 5'-to-3'-end-ligated viral
RNA extracted from serum.   In all 4 clones with GBV-B
sequences, the 5' UTR was truncated compared to the
published sequence (simon 1995a).   However, whereas one
clone (29c) had the exact 3' terminus previously
published by Simons et al. (Simons 1995a), the three
other clones (29a, 29b, 29d) had 150 additional terminal
nucleotides.   Compared with the published sequence, all
four clones had a single nucleotide insertion (C
residue) at position 9134.   Next, RACE using dC-tailing
only was performed on the 5' end of the negative-strand
RNA extracted from the liver homogenate.   All 11 clones
analyzed had additional sequences at the 3' terminus.
Compared with the published GBV-B sequence, two clones
(gb6, gb23) had 259 additional nucleotides, 8 clones
(gb9, gb19, gb20, gb21, gb24, gb25, gb30, gb35) had 236
additional nucleotides and 1 clone (gb8) had 232
additional nucleotides.   Moreover, all of these clones
had the insertion at position 9134.   The 3' UTR
sequences among the various clones were highly conserved
(Fig. 3).   To demonstrate that the terminal 22
nucleotides found only in clones gb6 and gb23 existed in
circulating viruses, RT-nested PCR was performed on 10-
fold serially diluted RNA extracted from the serum pool
GB 2/94 using an RT and external antisense primer
deduced from this sequence.   GBV-B RNA was detected at a
dilution of 10^{-7} and the sequence of the amplicon was
identical to the sequence recovered from the liver
homogenate.   Thus, the 3' UTR of GBV-B consists of a
short sequence of 30 nucleotides followed by a 11-24

- 26 -

nucleotide-long poly (U) tract (single C residues were
observed in GBV-B from the liver homogenate) and a 3'
terminal sequence of at least 309 nucleotides. The new
GBV-B 3' UTR sequence did not have significant homology
to any of the sequences deposited in the GenBank
database. A prediction of the secondary structure of
the 3' UTR sequence is shown in Figure 4. The most
notable feature of the secondary structure is a highly
stable stem-loop structure at the very 3' end consisting
of 47 nucleotides.

Example 3

The pGBB Clone of GBV-B is Infectious *in vivo*

The infectivity of RNA transcripts from the
consensus clone pGBB5-1 which encompassed only the
published GBV-B sequence (Simons 1995) was first tested.
Within the GBV-B sequence there were no deduced amino
acid differences and only 2 nucleotide differences (at
nucleotide positions 3475 and 7138) between the
consensus sequence of the cloning source (GBV-B 2/94)
and the sequence of pGBB5-1 clone. In addition, the 3'
UTR of pGBB5-1 had a deletion at nucleotide position
9134 and was missing the 3' terminal 259 nucleotides
(Fig. 3). Prior to transcription, the pGBB5-1 clone was
linearized at the *BamHI* site with digestion at the exact
GBV-B 3' terminus. The RNA transcripts from pGGB5-1
were injected into the liver of two tamarins (*S. mystax*
797 and 815). GBV-B RNA was not detected in weekly
serum samples collected during 17 weeks of follow-up.
As the susceptibility of these two tamarins to GBV-B was
subsequently demonstrated by experimental infection
using a GBV-B virus pool, the consensus clone pGBB5-1

- 27 -

which lacks the 3' terminal sequence of GBV-B is thus
not infectious *in vivo*.

        Next, the infectivity of RNA transcripts from
the full-length consensus GBV-B cDNA clone pGBB was
tested. The pGBB clone was identical to the pGBB5-1
clone except in the 3' UTR. Thus, in addition to a 5'
UTR of 445 nucleotides, an ORF of 8592 nucleotides
encoding 2864 amino acids and a 3' UTR of 103
nucleotides, the pGBB clone also contains an additional
259 nucleotides in its 3' UTR. pGBB was linearized at
the *Xho*I site which added an additional C residue at the
3' end of the transcribed GBV-B RNA. When RNA
transcripts from the pGBB clone were injected into the
liver of two tamarins (*S. mystax* 816 and 817), both
tamarins became infected with GBV-B with viremia at week
1 p.i. and peak viral titers of 10^8 GE/ml (Fig. 5). The
consensus sequence of PCR products of the complete ORF,
amplified from serum obtained during week 2 p.i. from
one tamarin (*S. mystax* 817), was identical to the
sequence of pGBB, including at the two positions which
differed from the consensus sequence of the cloning
source and from the published sequence of GBV-B (Table
1). By performing RT-PCR as desired above, it was
demonstrated that the very 3' terminal GBV-B sequence of
pGBB existed in the circulating viruses in this tamarin.
Within two weeks of the transfection both tamarins
developed hepatitis with dramatically elevated liver
enzyme levels (Fig. 5). Thus, the pGBB clone is
infectious *in vivo* whereas the clone pGBB5-1 which lacks
the last 259 nucleotides was not.

- 28 -

References

1.   Alter, H. J., Nakatsuji, Y., Melpolder, J., Wages, J., Wesley, R., Shih, J. W.-K. & Kim, J. P. (1997) The incidence of transfusion-associated hepatitis G virus infection and its relation to liver disease. *N. Engl. J. Med.* 336, 747-754.

2.   Alter, M. J., Gallagher, M., Morris, T. T., Moyer, L. A., Meeks, E. L., Krawczynski, K., Kim, J. P. & Margolis, H. S. (1997) Acute non-A-E hepatitis in the United States and the role of hepatitis G virus infection. *N. Engl. J. Med.* 336, 741-746.

3.   Bukh, J. & Apgar, C. L. (1997a) Five new or recently discovered (GBV-A) virus species are indigenous to New World monkeys and may constitute a separate genus of the *Flaviviridae.* *Virology* 229, 429-436.

4.   Bukh, J., Apgar, C. L., Engle, R., Govindarajan, S., Hegerich, P. A., Tellier, R., Wong, D. C., Elkins, R. & Kew, M. C. (1998b) Experimental infection of chimpanzees with hepatitis C virus of genotype 5a: genetic analysis of the virus and generation of a standardized challenge pool. *J. Infect. Dis.* 178, 1193-1197.

5.   Bukh, J., Apgar, C. L. and Purcell, R. H. (1997b) Natural history of GBV-A and GBV-B in   animal models: discovery of indigenous *Flaviviridae*-like viruses in several species of New World monkeys. In *Viral Hepatitis and Liver Disease* (Proceedings of the IX Triennial International Symposium on Viral Hepatitis and Liver Disease, Rome, Italy, 1996) (M. Rizzetto,   R. H. Purcell, J. L. Gerin, G. Verme, Eds.),   pp. 392-395. Edizione Minerva Medica, Turin, Italy.

6.   Bukh, J., Kim, J. P., Govindarajan, S., Apgar, C. L., Foung, S. K. H., Wages, J., Yun, A. J., Shapiro, M., Emerson, S. U. & Purcell, R. H. (1998a) Experimental infection of chimpanzees with hepatitis G virus and genetic analysis of the virus. *J. Infect. Dis.* 177, 855-862.

7.   Bukh, J., Miller, R. H. & Purcell, R. H. (1995) Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. *Semin. Liver Dis.* 15, 41-63.

8.   Deinhardt, F., Holmes, A. W., Capps, R. B. & Popper, H. (1967) Studies on the transmission of human viral hepatitis to marmoset monkeys: Transmission of disease, serial passages, and description of liver lesions. *J. Exp. Med.* 125, 673-687.

- 29 -

9.   Emerson, S. U., , Lewis, M., Govindarajan, S.,
Shapiro, M., Moskal, T. & Purcell, R. H.   (1992) cDNA
clone of hepatitis A virus encoding a virulent virus:
induction of viral hepatitis by direct nucleic acid
transfection of marmosets. *J. Virol.* 66, 6649-6654.

10.   Erker, J. C., Desai, S. M., Leary, T. P., Chalmers,
M. L., Montes, C. C. & Mushahwar, I. K. (1998) Genomic
analysis of two GB virus A variants isolated from captive
monkeys. *J. Gen. Virol.* 79, 41-45.

11.   Frolov, I., McBride, M. S. & Rice, C. M. (1998)
Cis-acting RNA elements required for replication of
bovine viral diarrhea virus-hepatits C virus 5'
nontranslated region chimeras. *RNA* 4, 1418-1435.

12.   Houghton, M. (1996) Hepatitis C viruses. In
"Fields Virology" (B. N. Fields, D. M. Knipe, P. M.
Howley, *et al.*, Eds.), Third ed., pp. 1035-1058.
Lippincott-Raven Publishers, Philadelphia.

13.   Kolykhalov, A. A., Feinstone, S. M. & Rice, C. M.
(1996) Identification of a highly conserved sequence
element at the 3' terminus of hepatitis C virus genome
RNA. *J. Virol.* 70, 3363-3371.

14.   Kolykhalov, A. A., Agapov, E. V., Blight, K. J.,
Mihalik, K., Feinstone, S. M. & Rice, C. M. (1997)
Transmission of hepatitis C by intrahepatic inoculation
with transcribed RNA. *Science* 277, 570-574.

15.   Lemon, S. M. & Honda, M. (1997) Internal ribosome
entry sites within the RNA genomes of hepatits C virus
and other flaviviruses. *Semin. Virol.* 8, 274-288.

16.   Linnen, J., Wages, J., Jr., Zhang-Keck, Z. Y., Fry,
K. E., Krawczynski, K. Z., Alter, H., Koonin, E.,
Gallagher, M., Alter, M., Hadziyannis, S., Karayiannis,
P., Fung, K., Nakatsuji, Y., Shih, J. W.-K., Young, L.,
Piatak, M., Jr., Hoover, C., Fernandez, J., Chen, S.,
Zou, J.-C., Morris, T., Hyams, K. C., Ismay, S., Lifson,
J. D., Hess, G., Foung, S. K. H., Thomas, H., Bradley,
D., Margolis, H. & Kim, J. P. (1996) Molecular cloning
and disease association of hepatitis G virus: A
transfusion-transmissible agent. *Science* 271, 505-508.

17.   Lu, H.-H. & Wimmer, E. (1996) Poliovirus chimeras
replicating under the translational control of genetic
elements of hepatitis C virus reveal unusual properties
of the internal ribosomal entry site of hepatitis C
virus. *Proc. Natl. Acad. Sci. USA* 93, 1412-1417.

- 30 -

18.   Muerhoff, A. S., Leary, T. P., Simons, J. N., Pilot-Matias, T. J., Dawson, G. J., Erker, J. C., Chalmers, M. L., Schlauder, G. G., Desai, S. M. & Mushahwar I. K. (1995) Genomic organization of GB viruses A and B: Two new members of the *Flaviviridae* associated with GB agent hepatitis. *J. Virol.* 69, 5621-5630.

19.   Purcell RH. (1993) The discovery of the hepatitis viruses. *Gastroenterology* 104, 955-963.

20.   Rice, C. M. (1996) *Flaviviridae*: The viruses and their replication, In "Fields Virology". (B. N. Fields, D. M. Knipe, P. M. Howley, et al., Eds.), Third ed., pp. 931-959. Lippincott-Raven Publishers, Philadelphia.

21.   Robertson, B., Myers, G., Howard, C., Brettin, T., Bukh, J., Gaschen, B., Gojobori, T., Maertens, G., Mizokami, M., Nainan, O., Netesov, S., Nishioka, K., Shin-i, T., Simmonds, P., Smith, D., Stuyver, L. & Weiner, A. (1998). Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. *Arch. Virol.* 143, 2493-2503.

22.   Scarcelli, E., Urbani, A., Sbardellati, A., Tomei, L., De Francesco, R. & Traboni, C. (1997) GB virus B and hepatitis C virus NS3 serine proteases share substrate specificity. *J. Virol.* 71, 4985-4989.

23.   Schlauder, G. G., Dawson, G. J., Simons, J. N., Pilot-Matias, T. J., Gutierrez, R. A., Heynen, C. A., Knigge, M. F., Kurpiewski, G. S., Buijk, S. L., Leary, T. P., Muerhoff, A. S., Desai, S. M. & Mushahwar I. K. (1995) Molecular and serologic analysis in the transmission of the GB hepatitis agents. *J. Med. Virol.* 46, 81-90.

24.   Simons, J. N., Pilot-Matias, T. J., Leary, T. P., Dawson, G. J., Desai, S. M., Schlauder, G. G., Muerhoff, A. S., Erker, J. C., Buijk, S. L., Chalmers, M. L., Van Sant, C. L. & Mushahwar, I. K. (1995a) Identification of two flavivirus-like genomes in the GB hepatitis agent. *Proc. Natl. Acad. Sci. USA* 92, 3401-3405.

25.   Simons, J. N., Leary, T. P., Dawson, G. J., Pilot-Matias, T. J., Muerhoff, A. S., Schlauder, G. G., Desai, S. M. & Mushahwar, I. K. (1995b) Isolation of novel virus-like sequences associated with human hepatitis. *Nature Med.* 1, 564-569.

26.   Tanaka, T., Kato, N., Cho, M.-J. & Shimotohno, K. (1995) A novel sequence found at the 3' terminus of

- 31 -

hepatitis C virus genome. *Biochem. Biophys. Res. Commun.* 215, 744-749.

27. Tellier, R., Bukh, J., Emerson, S. U., Miller, R. H. & Purcell, R. H. (1996) Long PCR and its application to hepatitis viruses: amplification of hepatitis A, hepatitis B, and hepatitis C virus genomes. *J. Clin. Microbiol.* 34, 3085-3091.

28. Yanagi, M., Purcell, R. H., Emerson, S. U. & Bukh, J. (1997) Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc. Natl. Acad. Sci. USA* 94, 8738-8743.

29. Yanagi, M., St. Claire, M., Shapiro, M., Emerson, S. U., Purcell, R. H. & Bukh, J. (1998) Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious *in vivo. Virology* 244, 161-172.

30. Yanagi, M., St. Claire, M., Emerson, S. U., Purcell, R. H. & Bukh, J. (1999) *In vivo* analysis of the 3' untranslated region of the hepatitis C virus after *in vitro* mutagenesis of an infectious cDNA clone. *Proc. Natl. Acad. Sci. USA* 96, 2291-2295.